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Color preferences and gastrointestinal-tract retention times of microplastics by freshwater and marine fishes

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Highlights

- Clown anemonefish ingested the most MP particles, zebrafish ingested the secondmost
- The most frequently ingested MP colors were red, yellow, and green in fish
- Clown anemonefish rely on color vision to recognize for certain MP colors
- MP excretion times varied widely among individuals of the same species



1	Color preferences and gastrointestinal-tract retention times of microplastics by
2	freshwater and marine fishes
3	
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15 Abstract (196 words)

16 We examined ingestion and retention rates of microplastics (MPs) by two freshwater 17 (Japanese medaka and zebrafish) and two marine fish species (Indian medaka and clown 18 anemonefish) to determine their color preferences and gastrointestinal-tract retention times. In our ingestion experiments, clown anemonefish ingested the most MP particles, 19 20 followed by zebrafish, and then Japanese and Indian medaka. Next, we investigated color 21 preferences among five MP colors. Red, yellow, and green MP were ingested at higher 22 rates than grey and blue MPs for all tested fish species. To test whether these differences 23 truly reflect a recognition of and preference for certain colors based on color vision, we 24 investigated the preferences of clown anemonefish for MP colors under light and dark 25 conditions. Under dark conditions, ingestion of MP particles was reduced, and color 26 preferences were not observed. Finally, we assessed gastrointestinal-tract retention times 27 for all four fish species. Some individuals retained MP particles in their gastrointestinal 28 tracts for over 24h after ingestion. Our results show that fish rely on color vision to 29 recognize and express preferences for certain MP colors. In addition, MP excretion times 30 varied widely among individuals. Our results provide new insights into accidental MP 31 ingestion by fishes.

32

33 Keywords: color preferences, microplastic, ingestion, fish

35 Introduction

In recent years, microplastic (MP) pollution (i.e., pollution involving plastic particles of 36 37 diameter < 5 mm) has become a serious environmental problem around the world 38 (reviewed by Bagaev et al., 2021; Galarpe et al., 2021; Li et al., 2016). Pinherio et al. 39 (2021) reviewed MP concentrations in marine and freshwater environments in various 40 countries including the United Kingdom, United States, Brazil, China, France, 41 Germany, India, Italy, Australia, Indonesia, and Japan. Furthermore, MP contamination 42 has been repeatedly identified in marine (reviewed by Hidalgo-Ruz et al., 2012) and 43 freshwater (reviewed by Yang et al., 2021) environments worldwide. Therefore, the 44 ecotoxicity of MP in aquatic organisms has become a topic of increasing concern 45 (Galloway and Lewis, 2016). 46 To date, various field and laboratory studies have examined the influence of 47 MP on fishes. In the laboratory, MP ingestion and uptake into the gastrointestinal tract has been observed in goldfish (Carassius auratus) (Grigorakis et al., 2017), zebrafish 48 49 (Danio rerio) (Kim et al., 2019), mummichog (Fundulus heteroclitus) (Ohkubo et al., 50 2020), and red seabream (Pagrus major) (Ohkubo et al., 2020). In addition, MP has 51 been identified in wild fish captured in the Lijiang River in China (Zhang et al., 2021), 52 the Mondego Estuary in Portugal (Bessa et al., 2018), the Xingu River in Brazil 53 (Andrade et al., 2019), the Pacific coast of Ecuador (Alfaro-Núñez et al., 2021), and the

northern Bay of Bengal in Bangladesh (Hossain et al., 2020). These results indicate that
MP ingestion by fishes is widespread.

56 Various colors of MP (e.g., transparent, black, blue, grey, green, red, purple, 57 and yellow) are present in marine (Courtene-Jones et al., 2017; Dai et al., 2018; Lusher 58 et al., 2014; Martin et al., 2017) and freshwater environments (Li et al., 2018; McNeish 59 et al., 2018; Su et al., 2016; Wu et al., 2020). Colored MP has also been detected in wild 60 fish (Bessa et al., 2018; Jawad et al., 2021; Kazour et al., 2020), suggesting that colored MP can potentially be mistaken for natural prey. If various marine and freshwater fishes 61 exhibit color preferences for MP ingestion, this would be important for understanding 62 63 the harmful influence of MP on wild fishes.

Another important factor for estimating the harmful influence of MP on fishes 64 is MP retention and excretion times. This information is needed to assess the extent to 65 66 which hazardous chemical substances adsorbed onto MPs can be absorbed into the body after ingestion. However, to our knowledge, there are only a few laboratory reports that 67 currently provide this data (i.e., for goldfish [Grigorakis et al., 2017], mummichog 68 69 [Ohkubo et al., 2020], and red seabream [Ohkubo et al., 2020]). More data are needed 70 on retention and excretion times in various fishes including both freshwater and marine 71 species, because MP is detected in both freshwater and marine environments. 72 In our study, we examine two freshwater fish species (Japanese medaka

73 Oryzias latipes and zebrafish Danio rerio) and two marine species (Indian medaka

74	Oryzias melastigma and clown anemonefish Amphiprion ocellaris). Japanese medaka
75	typically inhabits gently flowing rivers and waterways in Japan (Sakaizumi 1986;
76	Sakaizumi and Jeon 1987), and zebrafish inhabits moderately flowing to stagnant clear
77	waters in India, Pakistan, Bangladesh, Nepal, and Bhutan (Lawrence, 2007). Japanese
78	medaka and zebrafish are widely used as model freshwater fishes for ecotoxicological
79	studies under OECD test guidelines (OECD, 2012, 2013, 2019). Indian medaka inhabits
80	coastal marine waters and freshwater bodies in Pakistan, India, Myanmar, and Thailand
81	(Dong et al., 2014). Recently, Indian medaka has also become a common choice as a
82	model marine fish for ecotoxicological studies (He et al., 2021; Horie et al., 2019;
83	Wang et al., 2020; Zheng et al., 2020). Clownfish is found throughout the Indo-Pacific
84	(Camp et al., 2016; Chen and Hsieh., 2017; Chen et al., 2018).
85	First, we identify any preferences for MP color among Japanese medaka,
86	zebrafish, Indian medaka, and clown anemonefish under laboratory conditions. Next,
87	we estimate MP retention times for all four species. The findings of this study will be
88	useful for further estimation of MP ecotoxicity in these species.
89	
90	2. Materials and methods
91	All animal experiments were conducted according to the relevant national guidelines
92	(Act on Welfare and Management of Animals, Ministry of the Environment, Japan) and
93	the fish used in the present study were handled according to the animal care and use

94	guidelines of Kobe University. All animal experiments were approved by the
95	institutional animal care and use committee, Research Center for Inland Sea, Kobe
96	University (Permission number, 2021-04). Our research was also performed in
97 98	accordance with the ARRIVE guidelines.
99	2.1. Study organisms and MPs
100	We examined two freshwater fishes, Japanese medaka and zebrafish, and two marine

101 fishes, Indian medaka and clown anemonefish. Japanese medaka, zebrafish, Indian

102 medaka, and Clown anemonefish were bred at Kobe University (Hyogo, Japan). Both

103 marine and freshwater fishes were maintained at a water temperature of 25 ± 2 °C

104 (mean \pm SD) by using a recirculating system. Marine fishes were maintained at a

105 salinity of 32 PSU. Fish were raised under an artificial photoperiod of 12-h/12-h

106 light/dark. Marine species were kept in artificial seawater (Marine ART Hi; Osaka

107 Yakken Co. Ltd, Osaka, Japan). In this study, we used young fish and genetic sex was

108 unknown. Average total body lengths (mm) and wet body weights (mg) of each species

109 were as follows: 15.1 ± 0.1 mm and 30.0 ± 0.8 mg for Japanese medaka (age: 1-2

- 110 months after hatching); 12.6 ± 0.1 mm and 12.2 ± 0.4 mg for zebrafish (age: 1-2 months
- 111 after hatching); 16.7 ± 0.1 mm and 40.6 ± 0.8 mg for Indian medaka (age: 1-2 months
- 112 after hatching); and 31.1 ± 0.2 mm and 484.7 ± 11.9 mg for clown anemonefish (age: 4-
- 113 6 months after hatching).

- 115 USA) in five colors (red, blue, yellow, green, and gray). The average diameter and
- 116 particle density of each color was as follows. Red: $219.2 \pm 22.6 \mu m$, 0.98 g/cc; blue:
- 117 $279.0 \pm 17.0 \ \mu\text{m}$, 1.00 g/cc; yellow: $256.9 \pm 21.2 \ \mu\text{m}$, 1.00 g/cc; green: 253.6 ± 20.4
- 118 μ m, 0.98 g/cc; gray: 257.7 ± 21.7 μ m, 1.00 g/cc (Supplementary Figure 1).

- 120 2.2. MP ingestion assays (Experiments 1 and 2)
- 121 A flow chart summarizing the experimental procedure for all experiments (Experiments

122 1–4) is shown in Figure 1. For MP ingestion, we conducted two experiments: in

123 Experiment 1, we exposed each of the four fish species to a single color (red, blue,

124 yellow, green, or gray) of MP; in Experiment 2, we exposed each species to a mix of the

125 five MP colors to test for the presence of a color preference. Seven fish were placed in

126 5-L glass tanks filled with 4 L of water, and two replicate tanks were used for each

127 exposure (i.e., n = 14 per exposure).

Before establishing the nominal MP concentrations for exposures, we assessed the maximum MP ingestion amount for each fish species. The maximum MP ingestion test lasted for 4 h (until feeding behavior was no longer observed for four fish species). The Japanese medaka, zebrafish, and Indian medaka individuals that ingested the most MP particles in our assessment each ingested fewer than 100 particles, and the clown anemonefish that ingested the most MP particles ingested fewer than 1000 particles

134	(Figure 2). The weight of 1000 MP particles is approximately 10 mg. Because each 5-L
135	glass tank contained 4 L of water and seven fish in our exposure experiments, the
136	nominal MP concentration for Experiment 1 was set at 1.75 mg/L for Japanese medaka,
137	zebrafish, and Indian medaka, and 17.5 mg/L for clown anemonefish. For Experiment 2,
138	the concentration for each MP color was set at 1.75 mg/L (i.e., a total MP concentration
139	of 8.75 mg/L) for each of the three fish species. In case of clown anemonefish, the
140	concentration for each MP color was set at 17.5 mg/L (i.e., a total MP concentration of
141	87.5 mg/L).
142	Fish were placed into 5-L glass tanks 24 h prior to the start of each exposure
143	experiment, and were not fed during this time to ensure that their gastrointestinal tracts
144	would be empty. Each MP exposure test lasted for 4 h (until feeding behavior was no
145	longer observed for four fish species). After 4 h of exposure, fish were anesthetized
146	(MS-222 at a concentration of 200 mg/L) and their gastrointestinal tracts dissected, and
147	the number of MP particles in each gastrointestinal tract was counted under a
148	stereomicroscope (SZ61, Olympus).
149	
150	2.3. MP color-preference assays (Experiments 3)
151	In Experiment 3, we examined clown anemonefish (which ingested the most MP
152	particles in pretests prior to Experiment 1) to clarify whether fish rely on their color

153 vision to recognize MP colors. Exposure conditions were similar to those in Experiment

154	2. Seven fish were placed in 5-L glass tanks, two replicate tanks were used for each
155	exposure (i.e., $n = 14$ per condition), and the exposure concentration for each MP color
156	was set to 17.5 mg/L (i.e., total MP concentration of 87.5 mg/L).
157	Ten days prior to each exposure experiment, in order to reset the circadian
158	rhythm of the study animals, the husbandry schedules for the fish were reversed; i.e.,
159	feeding was performed in the dark, and water changes were performed in the light. Fish
160	were then placed into 5-L glass tanks 24 h prior to the start of each exposure
161	experiment, and were not fed during this time to ensure that their gastrointestinal tracts
162	would be empty. Next, MP exposure tests were carried out for 4 h (until feeding
163	behavior was no longer observed for clown anemonefish) under dark conditions. After
164	the 4-h exposure, fish were anesthetized (MS-222 at a concentration of 200 mg/L) and
165	their gastrointestinal tracts dissected, and the number of MP particles in each
166	gastrointestinal tract was counted under a stereomicroscope (SZ61, Olympus).
167	
168	2.4. MP retention in and excretion from the gastrointestinal tract (Experiments 4)
169	In Experiment 4, we examined the gastrointestinal tract retention and excretion times of
170	green MP (which was the most commonly ingested MP color in Experiment 1) in each
171	fish species. Exposure conditions were the same as in Experiment 1. After 4 h of
172	exposure, each fish was transferred from its 5-L glass exposure tank to a 1-L glass
173	beaker with a stainless-steel screen at the bottom for the excretion experiment.

174	The fish were confined under the stainless-steel screen (mesh size: 2 mm (width)*2 mm
175	(height)) (to prevent re-feeding for the excretion MP) so that the MP, once excreted,
176	could swim freely to the surface due to their low density. Once at the surface, they were
177	collected by using glass pipett and were counted under a stereomicroscope in directly
178	(SZ61, Olympus). A total of 14 glass beakers were prepared to accommodate the 14
179	experimental fish. After 1, 2, 4, 8, 16, and 24 h, the number of MP particles excreted
180	from each fish was counted, and all MP were removed from the 1-L beaker. After the
181	excretion test, fish were anesthetized (MS-222 at a concentration of 200 mg/L) and their
182	gastrointestinal tracts dissected, and the number of MP particles in each gastrointestinal
183	tract was counted under a stereomicroscope (SZ61, Olympus).
184	
185	2.5. Statistical analysis
186	All data were analyzed in Microsoft Excel. To analyze significant differences between
187	light and dark conditions, we used open source statistical software R (http://www.R-
188	project.org/) and the package Rcmdr (Fox and Bouchet-Valat 2018) to test for
189	homogeneity of variance using Bartlett's test (significance level, 5%), and tested for
190	significant differences using Steel's test. To analyze color preference in clown
191	anemonefish, we used Steel-Dwass multiple-comparison tests (significance level, 5%).
192	

3. Results

194 3.1. MP uptake by the four fish species

195	Table 1 shows a summary of the number of fish of each species in Experiment 1 that
196	ingested MP of each color. Zebrafish and clown anemonefish readily ingested MP of all
197	colors, but Japanese medaka and Indian medaka mainly ingested green MP. The
198	proportion of individuals that ingested MP of any color was highest for clown
199	anemonefish, followed by zebrafish, Japanese medaka, and Indian medaka.
200	Figure 2 shows the number of MP particles ingested by the four fish species. In
201	Japanese medaka, almost all individuals ingested fewer than 10 MP particles, with the
202	exception of one individual that ingested 31 blue MP particles and three individuals that
203	ingested 28, 29, and 77 green MP particles, respectively (Fig. 2a). In zebrafish, almost
204	all individuals ingested between 10 and 30 MP particles, and the most MP particles
205	were ingested by an individual that consumed 63 yellow MP particles (Fig. 2b). In
206	Indian medaka, almost all individuals ingested fewer than 10 MP particles, with the
207	exception of two individuals that ingested 12 and 24 red MP particles, respectively and
208	one individual that ingested 15 green MP particles (Fig. 2c). Clown anemonefish
209	ingested the most MP in total. Almost all individuals ingested more than 100 MP
210	particles, including one individual that ingested 744 red MP particles (Fig. 2d). There
211	were no consistent differences in MP intake by color across the four species.
212	



214	The number of fish that ingested MP in Experiment 2 was as follows: Japanese medaka,
215	4 of 14; zebrafish, 6 of 14; Indian medaka, 5 of 14; and clown anemonefish, 12 of 14
216	(Fig. 3). Figure 3 shows color preferences for MP ingestion in Japanese medaka (Fig.
217	3a), zebrafish (Fig. 3b), and Indian medaka (Fig. 3c); the results for clown anemonefish
218	are shown separately (Fig. 4b). Overall, the MP colors that were most frequently
219	ingested by Japanese medaka, zebrafish, Indian medaka, and clown anemonefish were
220	red, yellow, and green, and the least frequently ingested colors were blue and gray,
221	although significant differences could not analyze due to the number of MP particles
222	ingested were low level.
223	Next, we examined the presence or absence of color preferences (Fig. 4;
224	Experiment 2 and 3). In this experiment, we used clown anemonefish, which ingested
225	the most MP particles of the species examined in Experiment 1. Clown anemonefish
226	ingested fewer MP particles under dark conditions than under light conditions (Fig. 4a).
227	Moreover, whereas red, yellow, and green MP were preferred under light conditions
228	(Fig. 4b), no color preference was apparent under dark conditions (Fig. 4b). Results of
229	steel–Dwass multiple-comparison tests (significance level, 5%), showed that red MPs
230	was significantly more ingested in comparison to blue and gray MP under light
231	conditions (Fig. 4c), on the other hand, under dark conditions, no significant differences
232	were observed (Fig. 4c).

234	3.3. Time-course of MP retention in and excretion from the gastrointestinal tract
235	The results of the MP retention and excretion tests were as follows. Eight of 14
236	Japanese medaka (57%) excreted all of the MP contained in their gastrointestinal tracts
237	within 24 h (Figure 5); some residual MP remained in the gastrointestinal tracts after 24
238	h in the remaining six fish. Nine of 14 zebrafish (64%) excreted all of the MP contained
239	in their gastrointestinal tracts within 24 h (Figure 6); only one individual excreted less
240	than 90% of its ingested MP within the 24-h observation period. All 14 Indian medaka
241	excreted all of the MP contained in their gastrointestinal tracts within 24 h (Figure 7).
242	Nine of 13 clown anemonefish (69%) excreted all of the MP contained in their
243	gastrointestinal tracts within 24 h (Figure 8); only one individual excreted less than 20%
244	of its ingested MP within the 24-h observation period.
245	There was no consistent pattern of MP excretion among fish species, and the
246	excretion time varied widely among individuals of the same species. In addition, there
247	was no apparent difference in MP excretion time caused by the presence (clown
248	anemonefish) or absence (Japanese medaka, zebrafish, and Indian medaka) of a
249	stomach.
250	
251	4. Discussion

252 This study is the first to evaluate whether fish differentiate among five distinct colors of

MP under laboratory conditions and can preferentially ingest MP on this basis. 253

254	Our results on MP ingestion highlight the magnitude of variability in MP
255	ingestion among species and individuals. Recently, Ohkubo et al (2020) reported that
256	the number of MP particles (diameter, 250–300 μ m; color, yellow) identified in the
257	gastrointestinal tracts of mummichog was 352 ± 240 at an exposure concentration of 3
258	mg/L, and the number identified in red seabream was 41.8 ± 14.9 at an exposure
259	concentration of 0.9 mg/L. In the present study, the average number of MP particles of
260	the type examined in the above reports (diameter, 250–300 μ m; color, yellow) identified
261	in gastrointestinal tracts was 0, 25 ± 18 , and 1 in Japanese medaka, zebrafish, and
262	Indian medaka, respectively, at an exposure concentration of 1.75 mg/L; in clown
263	anemone fish the number was 215 \pm 198 at an exposure concentration of 17.5 mg/L.
264	Some of the species (Japanese medaka, zebrafish, and Indian medaka) do not possess
265	stomachs, and clown anemonefish do. However, the number of ingested MP particles
266	does not appear to depend strongly on the presence or absence of a stomach. Further
267	studies on other fish species could help identify commonalities between fish that are or
268	are not prone to accidental MP ingestion.
269	Our results on color preferences contrast with reports from the field. To date,
270	there have been numerous reports of plastics in the gastrointestinal tracts of wild fishes.
271	For example, blue and black MP filaments and blue, green, and black MP fragments
272	were frequently identified in Atlantic horse mackerel (Trachurus trachurus) from the

273 central Mediterranean Sea (Chenet et al., 2021). Along the southwest coast of the United

274	Kingdom, grey/transparent MP were the most frequently ingested by small-spotted
275	catshark (Scyliorhinus canicula), and green was the least frequently ingested (Morgan et
276	al., 2021). In the Adriatic Sea in Italy, black, tan, and blue MP fragments were
277	frequently found in the stomachs of Sardina pilchardus, and black and blue MP
278	fragments were frequently identified in the stomachs of Engraulis encrasicolus (Renzi
279	et al., 2019). By contrast, in our laboratory study, red, yellow, and green MP particles
280	were frequently observed in the gastrointestinal tracts of zebrafish, Indian medaka, and
281	clown anemonefish, and blue and gray particles were less common. This may indicate
282	that color preferences differ between field and laboratory conditions. Future studies on
283	the same species in the field and laboratory will clarify whether this is the case.
284	In general, vertebrates possess two types of photoreceptor cells: cones and rods.
285	Cone cells enable color vision during light conditions, and rod cells, which do not
286	distinguish among colors, are more sensitive under low-light conditions, meaning that
287	they are used most heavily in the dark. Bony fishes such as goldfish and zebrafish have
288	four spectral cone types comprising alternating rows of double cones with red (LWS)
289	and green (RH2) members and single blue (SWS2) and UV (SWS1) cones (Allison et
290	al., 2010; Baden and Osorio, 2019; Engström, 1960; Raymond et al., 1993). On the
291	other hand, the few shark species that have been studied only possess a single spectral
292	class of cone with LWS (Hart et al., 2011; Hart et al., 2020; Theiss et al., 2012),
293	indicating that they are almost certainly cone monochromats and do not possess color

294	vision (Schluessel et al., 2014). Recently, Mitchell et al (2021) reported that clown
295	anemonefish has four spectral cone types including LWS, RH2, SWS2, and SWS1, and
296	rhodopsin 1 (rod opsin, RH1). Therefore, this report and our results indicate that clown
297	anemonefish are able to recognize MP colors. Under light conditions, the species
298	showed a preference for red, yellow, and green MP, but under dark conditions, the
299	number of MP particles ingested declined and color preferences were not observed.
300	Various colors of MP have been identified in the field (Chenet et al., 2021; Morgan et
301	al., 2021; Renzi et al., 2019), and our study identified color preferences for MP
302	ingestion in clown anemonefish (a marine species). Further studies are needed to
303	determine the presence or absence of color preferences in other fish species to further
304	our understanding of MP ingestion in the field. In addition, color preference data could
305	help clarify whether fish are misidentifying MP as food.
306	To our knowledge, reports on MP retention and excretion times in fish in the
307	laboratory remain scarce, although there have been many reports of MP being identified
308	in the gastrointestinal tracts of wild fishes (Bessa et al., 2018; Jawad et al., 2021;
309	Kazour et al., 2020). Excretion times observed in the present study can be divided into
310	three patterns: (1) immediate excretion, in which most MP is excreted within the first 4
311	h; (2) gradual excretion, in which MP is excreted gradually over 24 h; and (3) delayed
312	excretion, in which most MP is excreted after 16 h. A previous study (Ohkubo et al.,
313	2020) on mummichog and red seabream also identified the above three patterns. In the

future, it will be necessary to investigate whether these three patterns apply to other fish species. In addition, why were there observed the difference of excretion patterns in same fish species? Various factors such as gender differences, health conditions, or fitness differences, etc. can be considered, therefore, further research is needed in the near future.

319 In contrast to previous reports, our results show that MP can remain in fish 320 gastrointestinal tracts for a prolonged period following ingestion. Ohkubo et al (2020) 321 reported that over 95% of ingested MP was excreted within 25 h in mummichog and red 322 seabream. Similarly, Naidoo and Glassom (2019) reported that only a few particles of 323 negligible mass remained 24 h after MP exposure in Ambassis dussumieri. By 324 comparison, we found that some Japanese medaka, zebrafish, and clown anemonefish 325 retained MP in the gastrointestinal tract even 24 h after MP ingestion. This indicates 326 that the time required for MP excretion does not depend on fish species, but on the 327 individual. In the future, we intend to examine excretion rates across a longer time 328 horizon to clarify the time required to discharge all ingested MP particles.

329

330 5. Conclusion

To assess the impact of MP on fish, it is essential to first understand accidental MP
ingestion and gastrointestinal-tract retention times. Ours is the first study to identify
color preferences among teleost fishes for MP ingestion, and also provides valuable data

334	on MP excretion times for both freshwater and marine fishes. The freshwater zebrafish
335	and marine clown anemonefish both ingested large numbers of MP particles compared
336	to Japanese and Indian medaka. Also, red, yellow, and green MP was more frequently
337	ingested than grey and blue MP overall. The number of MP particles ingested was
338	reduced, and color preferences were not observed under dark conditions in clown
339	anemonefish. These findings suggest that clown anemonefish rely on color vision to
340	recognize MP colors and express color preferences. MP excretion times varied widely
341	among fish of the same species, and some individuals still had MP particles remaining
342	in their gastrointestinal tracts more than 24 h after exposure. These findings provide
343	new insights into accidental MP ingestion by fishes.
344	
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350	

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511	Figures
	riguits

512	Figure 1.	Experimental	flow chart.	In Experim	nent 1, fish	from each	of the	four species
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- 513 were each exposed to a single color (red, blue, yellow, green, or gray) of microplastic
- 514 (MP). In Experiment 2, fish were exposed to a mix of the five MP colors under light
- 515 conditions. In Experiment 3, clown anemonefish were exposed to a mix of the five MP
- 516 colors under dark conditions. In Experiment 4, we determined gastrointestinal tract
- 517 retention and excretion times in each of the four fish species using green MP.
- 518

519 Figure 2. The number of microplastic (MP) particles in the gastrointestinal tracts of (a)

520 Japanese medaka, (b) zebrafish, (c) Indian medaka, and (d) clown anemonefish after

521 exposure to various MP colors. Circles show values for each individual.

Figure 3. The number of microplastic (MP) particles of various colors observed in the
gastrointestinal tracts of (a) Japanese medaka, (b) zebrafish, and (c) Indian medaka after

- 525 exposure to a mix of five MP colors. *X*-axis values show ID numbers for each fish: i.e.,
- 526 exposure tests were conducted on a total of four Japanese medaka, six zebrafish, and
- 527 five Indian medaka.
- 528

529 Figure 4. Color preferences of clown anemonefish under light and dark conditions. The530 number of fish that ingested MP was 12 of 14 in both light and dark conditions. (a) The

531	number of microplastic (MP) particles observed in clown anemonefish gastrointestinal
532	tracts under light (circles) and dark (triangles) conditions. (b) The number of MP
533	particles of various colors observed in the gastrointestinal tracts of clown anemonefish
534	under light conditions and dark conditions after exposure to a mix of five MP colors. X-
535	axis values show ID numbers for each fish: i.e., exposure tests were conducted on a
536	total of twelve individuals under light and dark conditions. (c) Box plot of the number
537	of MP particles of various colors observed in the gastrointestinal tracts of clown
538	anemonefish. *Values that are significantly different (Steel's test or Steel-Dwass
539	multiple-comparison tests; * $P < 0.05$)
540	
541	Figure 5. Microplastic (MP) excretion in Japanese medaka. Bars show numbers of
542	ingested MP particles remaining in fish gastrointestinal tracts at each sampling time,
543	and lines show % of total excreted MPs at each sampling time.
544	
545	Figure 6. Microplastic (MP) excretion in zebrafish. Bars show numbers of ingested MP
546	particles remaining in fish gastrointestinal tracts at each sampling time, and lines
547	show % of total excreted MPs at each sampling time.

- 549 Figure 7. Microplastic (MP) excretion in Indian medaka. Bars show numbers of
- 550 ingested MP particles remaining in fish gastrointestinal tracts at each sampling time,
- and lines show % of total excreted MPs at each sampling time.
- 552
- 553 Figure 8. Microplastic (MP) excretion in clown anemonefish. Bars show numbers of
- 554 ingested MP particles remaining in fish gastrointestinal tracts at each sampling time,
- and lines show % of total excreted MPs at each sampling time.
- 556
- 557 Supplementary Figure 1. Photographs of polyethylene microspheres in the five colors
- used in the study: (a) red, (b) blue, (c) yellow, (d) green, and (e) gray. Average particle
- 559 diameters for each color (f) were measured by using a stereomicroscope (SZ61,
- 560 Olympus). Error bars show \pm standard deviation (n = 100 per color).
- 561
- 562
- 563















Table

<u>C</u>	MD1.	Number of fish (percent of
Species	MP color	total)
	Red	0 of 14 (0%)
т	Blue	3 of 14 (21%)
Japanese	Yellow	0 of 14 (0%)
тедака	Green	9 of 14 (64%)
	Gray	3 of 14 (21%)
	Red	12 of 14 (85%)
	Blue	12 of 14 (85%)
Zebrafish	Yellow	12 of 14 (85%)
	Green	12 of 14 (85%)
	Gray	13 of 14 (92%)
	Red	3 of 14 (21%)
	Blue	0 of 14 (0%)
Indian medaka	Yellow	1 of 14 (7%)
	Green	5 of 14 (35%)
	Gray	0 of 14 (0%)
	Red	13 of 14 (92%)
Classe	Blue	12 of 14 (85%)
Clown	Yellow	13 of 14 (92%)
anemonensh	Green	14 of 14 (100%)
	Gray	10 of 14 (71%)

Table 1. The number of fish that ingested microplastic (MP) of various colors for the species Japanese medaka, zebrafish, Indian medaka, and clown anemonefish.



Study conception and design: Yoshifumi Horie; data collection: Konori Okamoto; analysis and interpretation of results: Miho Nomura and Hideo Okamura; draft manuscript preparation: Yoshifumi Horie and Konori Okamoto. All authors reviewed the results and approved the final version of the manuscript.

Declaration of interests

⊠The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: